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(57) Abstract

There is a provided a process for activating a kinase of a signalling pathway comprising treatment thereof with a phosphatase inhibitor. Moreover, we describe methods for screening candidate immunosuppressive and antiproliferative agents using thus activated kinases.

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Process for Activating a Kinase

The present invention relates to a method for producing an active form of a kinase involved in an insulin dependent signalling pathway, and to the use of the active kinase in screening techniques.

Background of the Invention

Protein phosphorylation and dephosphorylation are fundamental processes for the regulation of cellular functions. Protein phosphorylation is prominently involved in signal transduction, where extracellular signals are propagated and amplified by a cascade of protein phosphorylation and dephosphorylation. Two of the best characterised signal transduction pathways involve the c-AMP-dependant protein kinase (PKA) and protein kinase C (PKC). Each pathway uses a different second messenger molecule to activate the protein kinase, which, in turn, phosphorylates specific target molecules.

A novel subfamily of serine/threonine kinases has been recently identified and cloned, termed herein the RAC kinases (RAC-PK; Jones, et al. (1991) Proc. Natl Acad. Sci. USA 88, 4171-4175; Jones, et al. (1991) Cell Regulation 2, 1001-1009), but also known as PKB or Akt. RAC kinases have been identified in two closely related isoforms, RACα and RACβ, which share 90% homology at the gene sequence. Mouse RACα (c-akt) is the cellular homologue of the viral oncogene v-akt, generated by fusion of the Gag protein from the AKT8 retrovirus to the N-terminus of murine c-akt. Human RACβ is found to be involved in approximately 10% of ovarian carcinomas, suggesting an involvement of RAC kinases in cell growth regulation.

Another kinase implicated in cell growth control is S6 kinase, known as $p70^{S6K}$. S6 kinase phosphorylates the 40S ribosomal protein S6, an event which upregulates protein synthesis and is believed to be required in order for progression through the G_1 phase of the cell cycle. The activity of $p70^{S6K}$ is regulated by serine/threonine phosphorylation thereof, and it is itself a serine/threonine kinase. The $p70^{S6K}$ signalling pathway is believed to consist of a series of serine/threonine kinases, activating each other in turn and leading to a variety of

effects associated with cell proliferation and growth. RAC-PK is believed to lie on the same signalling pathway as $p70^{S6K}$, but upstream thereof.

As set forth in UK patent application 9523379.9 (Ciba-Geigy AG), filed on 16th November 1995, RAC-PK plays a major role in insulin-dependent signal transduction, which is important in a number of functions including the regulation of cell growth and glycogen metabolism. For example, glycogen synthase kinase-3 (GSK3), which is responsible for the activation of glycogen synthase, is a target for RAC-PK.

When isolated from natural sources, especially convenient sources such as tissue culture cells, RAC-PK and other signalling kinases are normally in the inactive state. In order to isolate active kinase proteins, it is necessary to stimulate cells in order to switch on the signalling pathway to yield active kinase. Moreover, when cells expressing kinase enzymes are used in kinase activity assays, it is necessary to employ activating agents prior to conducting the assay. Thus, cells are normally stimulated with mitogens and/or activating agents, such as IL-2, platelet-derived growth factor (PDGF), insulin, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Such agents are expensive and, when it is desired to produce active kinases or to activate cells in large amounts, the use of such agents is disadvantageous.

Screening of candidate compounds for activity as inhibitors of RAC-PK, or other signalling kinases in order to identify candidate immunosuppressive or antiproliferative agents requires a plentiful supply of kinase protein. Using modern day technology, it is possible to produce large quantities of virtually any desired protein in recombinant DNA expression systems. In the case of kinases such as those with which we are presently concerned, however, such systems are unsatisfactory because the proteins produced would be unphosphorylated and therefore inactive. There is therefore a requirement to identify a cost-effective way to produce phosphorylated kinase proteins which can be employed in screening procedures.

It is known (Janō *et al.*, (1988) Biochemistry, 85, 406-410) that vanadate can activate $p70^{S6K}$ itself. The mechanism of this activation, however, is not known. We have now found that vanadate acts generally on signalling kinases, activating them and preventing deactivation by phosphatases. Moreover, we have found that okadaic acid, a different class of

compound from vanadate which interacts with different proteins, may be used to similar effect.

Summary of the Invention

According to the present invention, there is provided a process for activating a kinase of a signalling pathway comprising treatment thereof with a phosphatase inhibitor. Moreover, the invention provides methods for screening candidate immunosuppressive and antiproliferative agents using thus activated kinases.

Detailed Description of the Invention

We have observed that modulation of RAC-PK activity appears to be effected by reversible phosphorylation, in which the equilibrium of the phosphorylation/dephosphorylation reaction is shifted in order to change the levels of active RAC-PK with respect to its inactive form. Build up of the active form may therefore be promoted by inhibition of the dephosphorylation reaction, achieved by treatment with a phosphatase inhibitor.

A surprising aspect of the present invention is that tyrosine phosphatase inhibitors, such as vanadate, are able to activate RAC-PK notwithstanding the fact that, as is disclosed herein, this kinase is activated by phosphorylation at threonine and serine residues.

It is known that vanadate activates $p70^{S6K}$. The invention accordingly does not extend to the use of vanadate to activate $p70^{S6K}$. However, the use of phosphatase inhibitors such as okadaic acid, which acts through a quite different mechanism, is part of the present invention.

As referred to herein, the signalling pathways are the activation cascades which ultimately regulate signal transduction and kinases of these pathways are kinases whose in-vivo targets include at least one entity which contributes to such signal transduction. Preferably, the signalling pathways of the invention are insulin-dependent signalling pathways, which are responsible for transduction of signals from insulin and other growth factors. Without in any way wishing to place any limitation on the present invention, one such a pathway is

believed to be triggered *in vivo* by binding of growth factors such as insulin and the like to their receptors, which stimulates *inter alia* phosphatidylinositol-3-OH kinase (PI-3K). PI-3K in turn directly or indirectly phosphorylates RAC-PK, which indirectly leads to the eventual phosphorylation of $p70^{S6K}$.

Treatment of kinases according to the invention in order to activate them requires the exposure of the kinase to a phosphorylating agent, such as another kinase of the signalling pathway, and the phosphatase inhibitor. This may be accomplished, for example, *in vitro* by

(a) incubating together a kinase of a signalling pathway, an agent capable of phosphorylating the kinase in order to activate it, and a phosphatase inhibitor; and

(b) purifying the kinase from the incubation mixture.

The phosphorylating agent should be effective to phosphorylate the kinase on residues which lead to activation thereof. In the case of RAC-PK, the phosphorylating agent advantageously targets serine and threonine residues.

Preferably, the phosphorylating agent is one or more kinases of the signalling pathway which act, in the presence of suitable activating factors, to phosphorylate and thereby activate the kinase of interest. Preferably, this is accomplished by recovering active kinase enzyme form phosphatase-inhibitor treated cells, which contain the required signalling pathway kinases.

In the context of the present invention, *in vitro* signifies that the experiment is conducted outside a living organism or cell. *In vivo* includes cell culture. Treatment of cells *in vivo* with phosphatase inhibitors is especially effective for the preparation of active RAC-PK. However, since RAC-PK and other signalling kinases, for example $p70^{S6K}$, are on the same pathway, activation of RAC-PK results in the activation of other kinases on the same signalling pathway, for example $p70^{S6K}$ itself. The invention therefore includes a method for activating kinases on signalling pathways in general, except for $p70^{S6K}$, especially where such kinases are downstream of RAC-PK in the pathway.

Cells which produce kinases which may be used in the present invention generally include any cell line of mammalian origin, especially fibroblast cell lines such as RAT-1, COS or NIH 3T3. Swiss 3T3 cells are particularly preferred. Where human cell lines are used, human embryonic kidney 293 cells are preferred.

Phosphatase inhibitors are agents which inhibit protein dephosphorylation by inhibiting the activity of phosphatase enzymes. A phosphatase has essentially the inverse activity of a kinase, and removes phosphate groups.

Examples of phosphatase inhibitors are vanadate and okadaic acid, with vanadate being the more effective agent in the case of RAC-PK. However, the action of vanadate is believed to be indirect, since it is a specific tyrosine phosphatase inhibitor and RAC-PK does not appear to be stimulated by tyrosine phosphorylation. Okadaic acid, on the other hand, which is known to act directly on phosphatase PP2A, appears to directly inhibit dephosphorylation of RAC-PK.

The use of other phosphatase inhibitors is envisaged and limited only by the suitability of such inhibitors for administration to the particular cell line being used. Vanadate and okadaic acid are believed to be generally applicable, but those of skill in the art will recognise that other phosphatase inhibitors are available and that their activity and suitability may easily be determined by routine empirical testing. For example, phosphatase inhibitors which may be suitable in the present invention include calyculin A, cantharidic acid, cantharidin, DTX-1, microcystin, nodularin and tautomycin. These and other phosphatase inhibitors are available commercially, e.g. from Calbiochem.

The phosphatase inhibitor is administered to cells in their normal growth medium, which may be serum free. Serum is itself observed to stimulate kinase activity, but is expensive and its function may be substituted by a phosphatase inhibitor according to the present invention. Suitable concentrations of phosphatase inhibitors include levels from 0.01mM to 10mM, preferably 0.1mM to 1mM. The most preferred concentration for vanadate is 0.1mM.

The method of the invention may comprise additional steps intended to isolate the desired active kinase from the cells in which it is produced. Such steps are conventional procedures familiar to those skilled in the art and may be substituted for equivalent

processes within the scope of the invention. The preferred process, however, comprises the steps of homogenising the cells, removing cell debris (for example by centrifugation) and separating the desired kinase by affinity purification.

Homogenisation may be carried out in a standard isotonic lysis buffer, advantageously containing a proteinase inhibitor such as phenylmethyl sulphonyl fluoride (PMSF) and a phosphatase inhibitor in order to inhibit deactivation of the kinase during the purification procedure. The cells are disrupted, thereby releasing the cytoplasmic and nuclear contents thereof into the lysis buffer.

Cell debris is then advantageously removed from the lysed cellular preparation, preferably by centrifuging the mixture in order to pellet all particulate matter. Only the soluble fraction remains in the supernatant.

The supernatant can then be subjected to standard protein purification techniques in order to isolate the kinase of interest if desired. Preferred methods, especially for relatively low volume preparations, involve affinity chromatography. Such techniques may employ an anti-kinase antibody or antiserum immobilised to a suitable matrix. Other immobilised binding agents, such as substrate analogues, may be employed.

Antibodies useful for immunoseparation of activated kinases according to the invention may be prepared according to techniques known in the art. In order to prepare a polyclonal serum, for example, an antigenic portion of the desired kinase, consisting of a peptide derived therefrom, such as a C-terminal peptide, or even the whole kinase, optionally in the presence of an adjuvant or conjugated to an immunostimulatory agent such as keyhole limpet haemocyanin, is injected into a mammal such as a mouse or a rabbit and antibodies are recovered therefrom by affinity purification using a solid-phase bound kinase or antigenic portion thereof. Monoclonal antibodies may be prepared according to established procedures.

Alternatively, and especially for larger scale preparations, separation procedures not involving affinity chromatography may be used.

For example, numerous methods are available in the art for separating polypeptides on the basis of size, such as chromatography and gel electrophoresis. Preferred are methods which perform a purification function as well as a size separating function, whilst not introducing unacceptable contaminants. Thus, methods such as step or continuous gradient centrifugation, particularly using sucrose gradients, dialysis techniques using controlled-pore membranes and membrane (Amicon) centrifugation are preferred. Especially preferred, however, is size exclusion chromatography, typically performed using porous beads as the chromatographic support. Size exclusion chromatography is, for example, described by Stellwagen, in Deutscher (1990) Guide to Protein Purification, Academic Press, Inc., San Diego, CA, USA, 317-328.

Alternative purification methods, described in general in Deutscher (1990), include chromatography based on separation by charge difference, such as ion exchange chromatography using an exchange group such as DEAE or CM bound to a solid phase packing material such as cellulose, dextran, agarose or polystyrene. Other methods include hydroxyapatite column chromatography (see, for example, Gorbunoff, (1985) Methods in Enzymology, 117, 370-380), and general affinity chromatography using glass beads or reactive dyes as affinity agents.

Advantageously, cation exchange chromatography may be employed, such that protein elution can be tailored to take into account the known or estimated pI of the kinase in question. The pI for any kinase may be determined experimentally, by isoelectric focusing. In this manner, it is possible selectively to elute from the cation exchange resin those proteins having a pI at or around that of the kinase, which results in a high degree of purification.

The invention further provides the use of an active kinase prepared according to the invention in a method for screening potential modulators of signalling pathways. Thus, the claimed method may comprise the additional step of exposing the kinase to a potential inhibitor and subsequently assessing the activity of the kinase in order to determine the effectiveness of the modulator.

The invention accordingly provides a method for screening candidate modulators of signalling pathways comprising:

(a) incubating together a kinase of a signalling pathway and a phosphatase inhibitor;

(b) adding a candidate modulator of the signalling pathway; and

(b) determining the activity of the kinase.

The exposure to the modulator may be performed on the activated or inactivated kinase either in a cell-free environment, optionally after purification of the kinase from the crude cellular preparation, or in situ in the cells which produce the kinase, after phosphatase inhibitor activation. Steps (a) and (b) may therefore be reversed, or conducted contemporaneously.

In step (a), especially if the assay is to be performed *in vitro*, an agent capable of phosphorylating the kinase may be added to the incubation mixture. Phosphatase inhibitors activate kinases by preventing dephosphorylation, so a phosphorylating agent will be required. Advantageously, the phosphorylating agent is a kinase of an insulin-dependent signalling pathway or an analogue thereof. Moreover, factors may be required to initiate or assist signal transduction in the signalling pathway. For example, it the compound being tested is a rapamycin analogue which binds FKBP, FKBP will be required in the incubation mixture.

Preferably, however, the procedure is carried out *in vivo* in cells containing kinases of the signalling pathway. In such an assay, the phosphatase inhibitor replaces serum or other agents previously employed as external stimulating agents to activate kinases of the signalling pathway.

The activity of the kinase may be assessed by means of a kinase activity assay, employing a substrate for the kinase. For example, myelin basic protein may be used, in accordance with established assay procedures. Physiological substrates, such as the 40S ribosomal subunit, or S6, may also be used. Alternatively, kinase activity may be assessed by determining the degree of phosphorylation of the kinase. Advantageously, phosphorylation on residues normally implicated in kinase activation is assessed. The identification of such residues, which is part of the present invention, is set forth below.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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